APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1994, p. 2180-2183 Vol. 60. No. 6
0099-2240/94/$04.00+0

Isolation and Identification of Environmental Mycobacteria in the *Mycobacterium bovis* BCG Trial Area of South India

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Received 12 December 1993/Accepted 5 April !991

The isolation profiles of environmental mycobacteria present in soil, water, and dust samples, and sputum samples of persons with symptoms of chest infection in the South lndian *Mycobacterium bovis* BCG (bacillus Calmette-Guérin) trial area were compared. Isolates belonging to the *Mycobacterium avium-intracellulare-scrofulaceum* complex were predominant in water, dust, and sputum samples and *Mycobacterium fortuitum*-complex organisms were predominant in soi1 samples irrespective of the season of the year.

The highly variable results with respect to the protective efficacy of *Mycobacterium bovis* BCG (bacillus Calmette-Gu érin) (range, 0 to 80%) (9) in major BCG trials have raised important questions regarding the immune mechanisms in tubeculosis, the nature of protection offered by BCG, and the rule played by environmental mycobacteria (29). In the southeastern United States, sensitization to nontuberculous mycobacteria (NTM) (4) has been found to be associated with the occurrence of these NTM in the environment (1, 23). Experimental work with animals has shown that infections with some NTM can offer a level of protection similar to that offered by BCG against tuberculosis (5, 17, 18, 20, 24) and that, depending on the nature of the NTM and the timing, this exposure can also enhance, mask, or interfere with the effect of subsequent BCG vaccination (25, 26). Thus, regional differences in environmental mycobacterial flora along with several other mechanisms could be responsible for the widely varying results of BCG trials (10) or could influence the course of subsequent infection with virulent tubercle bacilli (19).

In the large-scale BCG trial conducted in South India during 1968 to 1980, BCG did not offer any protection against the bacillary form of pulmonary tuberculosis (28). The study population in this that was was characterised by a high prevalence of tuberculous infection and disease and also by a very high prevalence of nonspecific sensitivity (16, 28, 30). Further, 20% of the NTM obtained from sputum samples of subjects in this area (in 1 year) belonged to the *Mycobacterium avium-intracellulare-scrofulaceum* (MAIS) complex (21). One of the several hypotheses put forward to explain the results of this trial suggests that prior exposure to NTM, which is present in the environment of this area, could have played a role in inodulating the immune response to subsequent BCG vaccination (29). Systematic investigations of the isolation and idenfication of mycobacteria from the environment of the area and comparison of such a mycobactcrial profile with that for sputum samples from subjects residing in the same area have not been done here so far. Studies conducted elsewhere have also been of limited scope; they generally highlight the prevalence of only a single mycobacterial species or complex in a particular environmental source (1, 8, 14, 15). In the present study, an attempt has been made for the first time to isolate and identify the various NTM species present in the soil, water, and house-dust of a pocket of the South Indian BCG trial arca.

Study area and collection of samples. Fifteen randomly selected villages in the BCG trial area near Madras formed the study area. From each village, 10 samples each of soil and water and 5 samples of dust were collected; in addition, 8 samples of water were collected from a reservoir in the area, once in January at the end of the monsoon season and once in June, in summer. The number of water samples collected in June was smaller because 49 of the 158 sources sampled in January had dried up.

From each site, soil was scraped up to a depth of about 3 cm with a sterile trowel and was collected in four McCartney bottles. Water was collected to fill two sterile 180-ml screw-cap glass bottles. Dust was swept up with a sterile brush onto 3 sterile piece of paper from sites such as the floor, roof-beams, and kitchen area and was transferred to a sterile McCartrney bottle. All the samples were protected from light during transportation to the laboratory and storage and were processed within 3 weeks of collection. Storage was at 4°C for water samples and at room temperature for soil and dust samples.

Processing of samples. Engback's method (6) was used for processing soil and dust samples and Engel's method (7), on the basis of our earlier experience (13), was used for water samples. Ten microliters of each of the processed samples was inoculated onto duplicate Lowcnstcin-Jensen (LJ) slopes, which were incubated at 30°C for soil and dust and at 37°C for water. The slopes were read for 8 weeks. Cultures positive for acid-fastness by Ziehl-Neelson staining were taken for further identification. Cultures that were positive with contamination were decontaminated with 1% cetrimide (12) to recover them as pure cultures of mycobacteria. All isolates obtained on primary cultures were subcultured on LJ medium, and only pure cultures were taken for identification. The tests in the preliminary identification, up to the level of Runyon's groups, included tests for growth rate, pigmentation. niacin production, growth on LJ medium containing para-nitrobenzoic acid (500 µg/ml), growth at 25°C, stability of catalase at 68°C for 20 min, and the Tween hydrolysis test. Detailed identification of all the isolates was made on the basis of methods of numerical taxonomy by using 19 biochemical tests as described previously (21). Isolates of NTM obtained from sputum samples which were collected at the same time-points as the environmental samples, from subjects included in a surveillance study in this area, were also included for comparison. These individuals

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TABLE 1. Culture results of soil, water and dust samples collected in January and June, 1992 and distribution of samples positivc for mycobacteria according to the number of species obtained per sample

Type of sample	Mo of collection	Total no. of samples	No. of samples positive for mycobacteria (%)	No. of isolates per sample					Total no. of isolates
				1	2	3	4	5	
Soil	January	150	112 (74.7)	72	33	6	0	1	161
	June	150	95 (633)	52	30	11	2	0	153
Water	January	158	102 (64.6)	56	29	15	2	0	167
	June	109	45 (41.3)	35	8	2	0	0	57
Dust	January	75	19 (25.3)	16	3	0	0	0	22
	June	75	14 (18.7)	11	3	0	0	0	17

were aged 10 years and above and had either symptoms of chest infection or histories of previous treatment

Statistical analysis. Chi-square and Fisher's exact tests were used to test for any association between the profiles, both within each type of sample at the two time-points and among the different types of samples at each time-point. Kappa and McNamer's tests were used for paired comparisons of samples from the same sites at the two time points (11).

Table 1 presents the culture results obtained from soil, water, and house-dust samples collected in January and June from the same sites in the study area. The highest number of mycobacterial isolates was obtained from soil samples; the lowest number was obtained from dust. The proportion of positive samples from soil, water, and dust was lower in June than in January. This reduction was statistically significant ($P = 0.046$) in the case of soil and highly significant ($P < 0.001$) in the case of water.

Among the positive samples, the majority yielded one or two species, with maximums of five and four species, respectively. yielded by positive soil and water samples. The proportion of samples yielding only one species was significantly increased in June as compared with January in the case of water samples ($P = 0.01$) but not in the case of soil samples ($P > 0.2$ and $P = 0.2$, respectively).

Table 2 presents the percentages of samples yielding different mycobacterial species. In a few cases, the isolates could not be identified up to species level with the procedures used; these have been grouped as "othcrs" among the slow growers and rapid growers.

Both soil and water samples yielded a greater proportion of pathogenic mycobacteria than nonpathogenic mycobactcria in both January and June. This was not seen in the case of sputum samples. Nonpathogenic mycobactcria and MAIS-complex organisms were isolated from a significantly greatcr ($P < 0.0001$ and $P < 0.01$, respectively) percentage of water samples in January than in June, even though the differences in the proportions of samples yielding pathogenic and nonpathogenic mycobacteria were not significant ($P > 0.2$ and $P = 0.1$, respectively) for soil or water.

The significant reductions in June, as compared with January, both in the proportions of soil, water, and dust samples positive for mycobacteria and in the numbers of MAIS-complex isolates and nonpathogens in water samples, suggest that climate could play an important role in the distribution of environmental mycobacteria. The high ambient temperature (ranging from 35 to 40°C) in the study area in June could be one of the reasons for the reduction in the number of positive samples among samples collected during this period.

The results indicate that in this area, the mycobacterial isolation profile for water most resembles the profile for sputum samples in certain aspects. For instance, from both. water and sputum, organisms belonging to the MAIS complex were the most frequently isolated in both January and June. There was no significant difference ($P = 03$) in the percentages of water and sputum samples yielding MAIS-complex organisms in June. Though there was a significant difference ($P = 0.02$) in the percentages of water and sputum samples yielding MAIS-complex organisms in January, it must be recalled that there was a significant reduction in June, as compared with January, in the proportion of water samples ($P < 0.01$) but not sputum samples ($P > 0.2$) yielding MAIS-complex organisms. The frequent occurrence of MAIS-complex organisms in sputum samples in the present study is in conformity with the findings of an earlier study in this area (21) in which MAIS-complex organisms were also the most frequently isolated (22.6% of all NM).

The comparison of isolation profiles from water and sputum samples also shows that isolates belonging to the *Mycobacterium fortuitum* complex are few in both cases. The proportions of water and sputum samples yielding *M. fortuitum*- complex organisms were not significantly different in either January ($P = 0.3$) or June ($P = 0.2$).

The isolation profile for soil is markedly different from the isolation profiles for water and sputum. Isolates belonging to the *M. fortuitum* complex were predominant in soil. *M. fortuitum* -complex isolates were yielded by significantly higher proportions of soil samples ($P < 0.0001$) than of water or sputum samples in both January and June. On thc other hand, the proponion of samples yielding MAIS-complex isolates was significantly lower for soil samples than for water and sputum samples in both January and June ($P < 0.0001$ and $P = 0.02$, respectively, for water and $P < 0.01$ and $P< 0.0001$, respectively, for sputum). Further, isolates belonging to *Mycobacterium asiaticum*, *Mycobacterium gordonae*, and *Mycobacterium triviale* were obtained from water and sputum samples alone.

It has been earlier proposed that inhalation of aerosols containing *M. avium*, *Mycobacterium kansasii*, and *Mycobacterium xenopi* generated in water systems, showers, and sinks may cause infection or colonization of the respiratory tract (2, 3). After experiments to simulate natural aerosolization of *M. intracellulare* and *M. scrofulaceum*, Parker et al. (22) reported that *M. intracellulare* was significantly more concentrated in water droplets than *M. scrofulaceum* and that the water-to-air pathway could be one of the means of human infection by NTM. The similarity in the isolation profiles of mycobacteria from water and sputum samples in the present study also suggests this possibility.

The isolation profile for dust is interesting, again because of certain similarities with the profile for sputum samples. In an earlier study, *M. fortuitum*, *M. gordonae*, and *Mycobacterium nonchromogenicum* were found to be the most frequently isolated species from dust, followed by *M. intracellulare* and *M. scrofulaceum* (27). According to the authors, dusts were the source of mycobacteria occurring in sputa as casual isolates,

TABLE 2. Percentages of environmental and sputum samples yielding different mycobacterial species

Species	Sample type							
	Soil		Water		Dust		Sputum	
	Jan[a]	June	Jan	June	Jan	June	Jan	June
Rapid growers								
Potential pathogens								
M. fortuitum complex	48.0	37.3	3.2	2.8	6.7	2.7	6.2	5.9
Total	48.0	37.3	3.2	2.8	6.7	2.7	6.2	5.9
Nonpathogens								
M. diernhoferi	3.3	0.7	8.2	1.8	1.3	0.0	21.9	7.7
M. gadium	7.3	14.7	3.8	0.9	0.0	1.3	4.2	1.0
M. parafortuitum	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
M. phlei	3.3	2.7	0.6	0.9	0.0	0.0	9.4	10.1
M. smegmatis	4.0	0.3	3.2	1.8	0.0	0.0	5.2	8.0
M. thermoresistible	2.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4
M. vaccae	3.3	2.7	5.1	2.8	0.0	0.0	5.2	4.2
Total	23.9	24.1	20.9	8.2	1.3	1.3	45.9	32.4
Others (rapid)	12.0	18.7	1.3	2.8	1.3	1.3	3.1	17.1
Slow growers								
Potential pathogens								
MAIS complex	16.7	16.7	50.0	29.4	13.3	17.3	34.4	35.0
M. asiaticum	0.0	0.0	2.5	0.0	0.0	0.0	0.0	1.4
M. kansasii	0.0	0.7	2.5	0.9	0.0	0.0	0.0	0.0
M. malmoense	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3
M. szulgai	0.0	0.7	1.3	2.8	0.0	0.0	0.0	0.7
M. xenopi	0.0	0.0	0.0	0.0	1.3	0.0	2.1	0.0
Total	16.7	18.1	56.3	33.1	14.6	17.3	36.5	37.4
Nonpathogens								
M. flavescens	0.7	0.7	0.6	0.0	1.3	0.0	0.0	0.7
M. gastri	1.3	0.0	0.0	0.0	0.0	0.0	2.1	0.3
M. gordonae	0.0	0.0	3.8	1.8	0.0	0.0	4.2	0.7
M. nonchromogenicum	0.0	0.7	4.4	0.0	0.0	0.0	4.2	0.7
M. terrae	4.0	2.0	7.6	1.8	0.0	0.0	2.1	5.9
M. triviale	0.0	0.0	0.6	0.0	0.0	0.0	0.0	1.4
Total	5.0	3.4	17.0	3.6	1.3	0.0	12.6	9.7
Others (slow)	0.7	0.7	7.0	1.8	4.0	0.0	0.0	0.0
Total no. of samples	150	150	158	109	75	75	96	286
Total no. of isolates	161	153	167	57	22	17	100	294
Total no. of species identified	12	12	15	11	5	3	12	17

[a] Jan, January.

and the pathogenic mycobacteria in dusts were more likely to survive in the human respiratory tract in the present study, MAIS-complex organisms were the predominant isolates from dust as well as from sputum and water. Isolates belonging to the *M. fortuitum* complex were less frequently isolated from dust. A few other species were also obtained from dust; they include *Mycobacterium diernhofen*, *Mycobacterium flavescens*, and *Mycobacterium xenopi*.

Thus, this study indicates that in the South Indian BCG trial area, organisms belonging to the MAIS complex are the predominant nontuberculous mycobacterial species in water and dust samples from the environment and in sputum samples from objects residing in the same area, while organisms belonging to the *M. fortuitum* complex are predominant in soil. It would be of interest to further investigate the isolates belonging to these two complexes.

This study was carried out with the help of a research fellowship granted by the Indian Council of Medical Research (ICMR) to T. Kamala, an ICMR junior research fellow.

We acknowledge the help rendered by the staff of the Bacteriology Department, Epidemiology Unit, and the Statistics Department of the Tuberculosis Research Centre (one of the permanent Institutes of ICMR), Madras, in the present investigation. We are grateful to D. B. Lowrie, National Institute of Medical Research, Mill Hill, London, for his interest in this study.

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